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Promotion and Inhibition of Calcium Carbonate Crystallization *In Vitro* by Matrix Protein From Blue Crab Exoskeleton

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Abstract. Soluble organic matrix isolated from dorsal carapaces of the blue crab, *Callinectes sapidus*, inhibited $CaCO_3$ crystallization when free in solution. Immobilized matrix complexes, prepared by crosslinking soluble matrix to decalcified crab carapace, promoted $CaCO_3$ formation in that crystallization in the presence of the immobilized soluble matrix complexes began sooner than in solution controls. In the experimental treatments, deposition of crystals occurred only within the complexes and not in the crystallization solutions. Chitin, a polymer of N-acetyl-D-glucosamine, and chitosan, a deacetylated chitin, which are both insoluble products of the organic matrix of the crab carapace containing little to no matrix protein, did not promote $CaCO_3$ crystallization. Complexes of immobilized polyanionic synthetic peptides on chitosan also promoted $CaCO_3$ crystallization. Addition of a hydrophobic tail (Ala_8) to the polyanionic peptide (Asp_{20}) reduced the rate of promotion, possibly because the hydrophobic tail formed a diffusion barrier around crystal nuclei growth sites, suppressing interactions of nascent crystal nuclei with ions in the bulk solution.

Introduction

The majority of biominerals are formed by an organic matrix-mediated process (Lowenstam, 1981). Organic matrix, extracted by dissolving biomineral in EDTA or dilute acid, is composed of two components that are separated based on solubility. The soluble component—designated soluble matrix (SM)—is typically composed of anionic proteins, and the insoluble component—designated insoluble matrix (IM)—often contains more hydrophobic proteinaceous material that may be crosslinked (Crenshaw, 1972, 1980, 1982; Krampitz *et al.*, 1976, 1983; Weiner, 1979; Weiner *et al.*, 1983; Wheeler and Sikes, 1984; Wheeler *et al.*, 1988a, b). In the crab exoskeleton, the IM may be primarily chitin (Hunt, 1970; Welinder, 1974). The association of soluble and insoluble matrix molecules with each other and with crystals affect how biomineralization is regulated. Of particular interest is inhibition and promotion of crystal growth (Crenshaw, 1982; Mann, 1983; Weiner, 1986; Sikes and Wheeler, 1988a; Wheeler and Sikes, 1984, 1989).

Soluble matrix, when free in solution, inhibits *in vitro* $CaCO_3$ crystallization, but when attached to an insoluble matrix framework it may promote crystallization (Crenshaw *et al.*, 1988; Sikes and Wheeler, 1988a; Wheeler *et al.*, 1988b; Wheeler and Sikes, 1984, 1989; Campbell *et al.*, 1989; Linde *et al.*, 1989). These apparently opposing functions have been explained by the immobilized-matrix/crystal nucleation (IC) hypothesis (Sikes and Wheeler, 1988a; Wheeler and Sikes, 1989), which states that, when matrix is immobilized, it binds to one portion of the surface of the crystal nucleus reducing surficial energy—the energy involved with interactions such as dissolution, or ion exchange, at the surface of the crystal nucleus. This in turn lowers the energy of crystal nucleus formation, the energy required for attaining a stable nucleus upon which crystal growth ensues. If free in solution, however, matrix may inhibit growth of the crystal nucleus, or a crystal surface, by binding to growth sites over the entire nucleus or crystal surface (Sikes and Wheeler, 1988a).

We have designed two *in vitro* systems for evaluation of aspects of the IC hypothesis. One system, referred to as the crab carapace system, enabled us to study the initiation of crystallization by sampling noncalcified and

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calcified cuticle at sequential stages of the molt cycle (Roer and Dillaman, 1984; Roer *et al.*, 1988). In this study, decalcified samples of calcified and noncalcified carapaces at various molt stages from the blue crab, *Callinectes sapidus*, were tested as immobilized matrix complexes in *in vitro* $CaCO_3$ crystallization assays.

The second system used chitosan, a deacetylated chitin. Chitosan provides an insoluble support with reactive amine groups at which various specific matrix molecules can be immobilized. For example, matrix molecules isolated from biominerals have polyanionic and hydrophobic regions. The hydrophobic regions may occur as part of the C-terminus (Schlesinger and Hay, 1977, 1986; Hay *et al.*, 1979; Butler *et al.*, 1983; Rusenko, 1988, Rusenko *et al.*, 1990) or the N-terminus (Gorski and Schimizu, 1988). Anionic regions of matrix molecules may be active sites for crystal binding, whereas the hydrophobic regions may act as diffusion barriers around crystal growth sites (Sikes and Wheeler, 1988a; Wheeler and Sikes, 1989). Therefore, peptide-chitosan complexes were tested as promoters of $CaCO_3$ crystallization, considering that polyanionic peptides having a hydrophobic C-terminus might have a suppressed ability to promote crystal formation.

Materials and Methods

Collection and maintenance of crabs

Blue crabs (*Callinectes sapidus*) were collected from the Mobile Bay area. Some crabs were killed immediately and others were maintained individually in Instant Ocean (specific gravity = 1.005, 22–24°C) and fed chicken liver three to four times per week. Samples of the noncalcified pre-ecdysial cuticle located underneath the old exoskeleton were obtained, as were samples of calcified cuticle after ecdysis. All crabs were staged by the method of Freeman *et al.* (1987).

Crab carapace studies

Dorsal crab carapaces, removed from the crab at various stages, were trimmed of the ragged edges, and cleaned with a soft brush and tap water. Soluble matrix was obtained by grinding approximately five intermolt carapaces (stage $C_{3/4}$) in an electric mill. A slurry of 10 g of powdered material was placed in dialysis tubing (20,000 MW cutoff; Sigma) open to the air to allow the release of carbon dioxide during dissolution of $CaCO_3$. The preparation was dialyzed against 4 l of 2% acetic acid for 24 h, followed by dialysis with two changes of 2 l of deionized distilled water (12 h for each change). Soluble matrix was separated from IM by centrifugation at 30,000 × *g* for 25 min. The SM-containing fraction was adjusted to neutrality. The concentration of protein in SM was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Immobilized matrix complexes were obtained by placing dorsal crab carapaces in 250 ml of 2% acetic acid (pH 2.4) or 10% ethylenediamine-tetraacetate (EDTA) at pH 8.0 for 48 h with magnetic stirring. Decalcification by these methods removed greater than 99% of the calcium, based on atomic absorption analysis (Perkin-Elmer Model 460) of calcified and decalcified samples treated with 1 *N* HCl. The residual calcium was presumably bound to ion exchange sites. Crosslinking of solubilized protein to the samples of carapace was accomplished by including 4% formaldehyde in the decalcification solutions.

Selected decalcified carapaces were boiled in 2 *N* NaOH for 10–15 h until pigmentation was lost. This treatment removed covalently bound protein, leaving only the IM framework, or chitin (Welinder, 1974). The decalcified carapaces were washed in 2 l of deionized distilled water for 48 h with one change of water at 24 h. Carapaces were stored in deionized distilled water. Samples of these carapaces (hereafter referred to as coupons) were cut with a knife blade against a glass plate. The size of the coupons, measured with a ruler as they were cut to be 1.7 cm × 3.0 cm (5.1 cm^2), was chosen to provide more than one coupon per carapace and so that a coupon would fit in the reaction vessel. Coupons were stored in deionized distilled water in Petri dishes.

The solutions that had been used for decalcification and crosslinking SM onto the carapace, as well as controls consisting of unused solutions, were dialyzed against distilled water with 1000 MW cutoff dialysis tubing, and then lyophilized (VirTis freeze dryer). Protein per milligram lyophilized material was determined by the method of Lowry *et al.* (1951). The amount of solubilized protein that was crosslinked to the carapace was determined as the difference in the protein left in the formaldehyde-containing solutions compared to those without formaldehyde.

Peptide synthesis

Peptides used in this study were made with an automated solid-phase peptide synthesizer (Applied Biosystems Model 430A) as described by Sikes and Wheeler (1988b). They were synthesized with the alanine residues located at the C-terminus; thus, the peptides were crosslinked to chitosan (as described below) at the N-terminal aspartic acid residue via glutaraldehyde, so that the C-terminal hydrophobic tail (Ala_8) would be free to interact with the crystallization solution. Sequences of chosen peptides were confirmed by amino acid analysis using a PICO-TAG amino acid analysis system (Waters) with a Varian VISTA 5500 High Performance Liquid Chromatography system. The synthetic peptides were also se-

quenced with an automated protein sequencer (Applied Biosystems Model 477A), with an on-line amino acid analyzer (Applied Biosystems Model 120A) using standard cycles of Edman degradation, with the amino acid residues identified as phenylthiohydantoin (PTH) derivatives (Applied Biosystems).

Peptide-chitosan studies

Chitosan was prepared by refluxing decalcified crab carapaces at intermolt (stage $C_{3/4}$) in 12.5 *N* NaOH (approximately four trimmed carapaces in 140 ml) for 27 h (Wu and Bough, 1978). The chitosan pieces were then washed in 2 l of deionized distilled water for 48 h, with one change at 24 h, and were stored in deionized distilled water. Coupons of chitosan were also cut with a knife blade against a glass plate and stored in deionized distilled water in Petri dishes. Chitosan was quantified as the percent free amine (wt/wt) of chitosan, as determined by base titration (Filar and Wirick, 1978).

Peptides were crosslinked onto chitosan coupons following the method of Masri *et al.* (1978). Peptides were added to 22 ml of 0.05 *M* sodium acetate at pH 5.80 ± 0.02 in a 30 ml screwcap bottle in a water bath maintained at 20.00 ± 0.02°C (VWR 1155 Constant Temperature Circulator) at a concentration of 100 μg peptide/ml. A chitosan coupon (5.1 cm^2) and 70.4 μl of 2.5% glutaraldehyde (Sigma) were added, and these components were gently stirred for 1 h. This quantity of glutaraldehyde successfully immobilized the peptides without rendering the coupon too brittle for later manipulation. The coupon was tied with a string in a cheesecloth bag and rinsed in two changes of 2 l of deionized distilled water (12 h for each change). Coupons were stored in Petri dishes. The concentration of the peptide in a 2-ml sample of the crosslinking solution, before and after the procedure, was determined by the hydrolysis/ninhydrin assay (Stewart and Young, 1984). The difference between the samples reflects the amount of peptide crosslinked onto chitosan. Control experiments were performed as described above without chitosan coupons.

$CaCO_3$ crystallization assay

Crystal formation was monitored by the change in pH of a solution supersaturated with respect to calcium and carbonate. The assay began with an induction period of relatively constant pH, during which crystal nucleation occurs, and then continues with a rapid decrease in pH indicating crystal growth. The pH was continuously monitored (Orion 901 pH meter) and recorded (Perkin-Elmer, Model 56 chart recorder), with periodic manual notation of exact pH values on the curves. The incubation medium included 29.1 ml of artificial seawater (ASW: 0.5 *M* NaCl and 0.011 *M* KCl) in a round bottom flask placed in a recirculating water bath (VWR 1155 Constant Temperature Circulator) maintained at 20.00 ± 0.02°C, to which 0.3 ml of 1.0 *M* $CaCl_2 \cdot 2H_2O$ was added to obtain 10 m*M* calcium. Next, SM (Lowry protein assay) or peptide was added and stirred for 10 min. Finally, 0.6 ml of 0.4 *M* $NaHCO_3$ was added to yield 8 m*M* dissolved inorganic carbon (DIC), and the solution was titrated to a pH of 8.30 ± 0.02 with microliter quantities of 1 *N* NaOH to begin the assay.

The above system was modified to establish longer induction times to be used for testing promotion of crystallization. A coupon, patted dry three times with a lab tissue (Kimwipe) and weighed (wet weight), was suspended in 53.84 ml of ASW in a round bottom flask at 20.00 ± 0.02°C (VWR 1155 Constant Temperature Circulator). The coupon was suspended by a string attached to a brass pin that held the coupon. Then, 0.55 ml of 1.0 *M* $CaCl_2 \cdot 2H_2O$ and 0.61 ml of 0.5 *M* $NaHCO_3$ were added to a concentration of 10 m*M* calcium and 5.5 m*M* DIC, respectively. The coupon was carefully placed in the solution so that it was not in contact with the electrode or the stir bar. As above, the pH was raised to 8.30 ± 0.02 to begin the experiment.

Verification of $CaCO_3$ deposition

The amount of calcium deposited on coupons, before and after the crystallization experiments, was measured by atomic absorption spectrophotometry (Perkin-Elmer, Model 460). Coupons were removed from the crystallization solution and vortexed in 10 ml of 1 *N* NaOH to remove extraneous calcium, while preventing dissolution of the $CaCO_3$ present on the coupons. The coupons were allowed to air dry before they were vortexed in 10 ml of 1 *N* HCl. The coupons were then dried at room temperature and weighed (dry weight). The acid solutions were then aspirated through an air/acetylene flame, the calcium detected with a calcium lamp, and the concentration determined from a standard curve.

The amount of carbonate removed from solution, which resulted in the observed downward pH drift during crystallization, was calculated using the equilibrium equations for dissolved inorganic carbon (Stumm and Morgan, 1981, pp. 186 and 197). These amounts were compared to the measurements of calcium deposition by atomic absorption spectrophotometry.

In addition, the presence of crystals on the coupons was verified visually by polarized microscopy. Pieces of coupons were viewed under polarized light before and after crystallization experiments, and with and without the addition of 0.01 *N* HCl to cover the samples and dissolve any $CaCO_3$ crystals that may have formed.

Table I

Inhibition of $CaCO_3$ crystallization by crab soluble matrix added at 0.10 μg/ml, and by various peptides added at 0.05 μg/ml

	n	Induction period (min)	Maximum growth rate (pH/min)
Control	14	6.4 ± 1.0	0.050 ± 0.007
Extract 1	5	109.0 ± 57.2	0.046 ± 0.006
Extract 2	9	43.8 ± 11.6	0.053 ± 0.004
Extract 3	3	49.6 ± 10.0	0.058 ± 0.004
Asp_{20}	3	274.6 ± 42.7	0.009 ± 0.012
$Asp_{20}Ala_8$	6	270.0 ± 76.4	0.006 ± 0.006
Asp_{40}	3	298.0 ± 79.2	0.009 ± 0.012
$Asp_{40}Ala_8$	3	290.0 ± 69.3	0.003 ± 0.002

Extracts 1, 2 and 3 were prepared separately from different samples of Stage $C_{3/4}$ carapaces.

Means ± standard deviations.

Statistics

Statistical analysis of data was performed using True Epistat (Tracy L. Gustafson, Epistat Services, Richardson, Texas).

Results

Soluble matrix from the blue crab carapace inhibited $CaCO_3$ crystallization, as indicated by a longer induction period (Table I). The inhibitory activity of the extracts varied, possibly reflecting the differing relative amounts of the various components of the crude preparations and the variation in the individual carapaces used for each isolation. In fact, gel chromatography revealed that these preparations differed in average relative molecular weight (Gunthorpe, 1989). Polyaspartate of 20 and 40 amino acid residues, and their counterparts with an hydrophobic (Ala_8) tail located at the C-terminus, inhibited $CaCO_3$ crystallization.

Promotion of crystallization, indicated by a shortened induction period, was shown when coupons of various decalcified carapaces were suspended in the crystallization solution (one-way ANOVA, $P < 0.01$, Table II) with the exception of pre-ecdysial cuticle. Soluble matrix was crosslinked to the carapace during decalcification in the presence of formaldehyde. There was a difference of 5.89 ± 1.08 mg protein/mg calcified carapace (S.D., n = 8; Stage D) and 6.40 ± 0.50 mg protein/mg calcified carapace (S.D., n = 6; Stage $C_{3/4}$) between the decalcification so-

Table II

The effect of fixation on promotion of $CaCO_3$ crystallization by coupons (5.1 cm^2) of decalcified crab carapaces at various stages of the molt cycle

Treatment	n	Wet weight (mg)	Dry weight (mg)	Induction period (min)	Maximum growth rate (pH/min)
Control	28	—	—	16.2 ± 2.9	0.0280 ± 0.0040
Formaldehyde					
0.074%	3	—	—	21.8 ± 5.1	0.0280 ± 0.0030
Chitin	3	221.0 ± 88.6	43.5 ± 14.0	22.3 ± 7.5	0.0280 ± 0.0020
2% acetic acid					
Stage D	4	258.5 ± 43.5	46.8 ± 10.0	9.3 ± 4.2	0.0024 ± 0.0012
Stage $C_{3/4}$	2	280.0 ± 19.0	55.0 ± 2.8	4.8 ± 1.4	0.0029 ± 0.0001
New cuticle					
Pre-ecdysial	3	38.0 ± 8.2	4.6 ± 0.3	15.3 ± 0.3	0.0005 ± 0.0001
Post-ecdysial	5	56.8 ± 3.0	9.5 ± 1.4	2.5 ± 1.0	0.0028 ± 0.0005
4% Formaldehyde in 2% acetic acid					
Stage D	5	225.7 ± 25.9	42.5 ± 3.9	4.5 ± 2.2	0.0035 ± 0.0070
Stage $C_{3/4}$	3	229.1 ± 18.4	64.3 ± 0.5	3.5 ± 1.4	0.0027 ± 0.0030
New cuticle					
Pre-ecdysial (F)	3	57.6 ± 1.5	11.8 ± 0.6	1.6 ± 0.8	0.0046 ± 0.0009
Post-ecdysial	5	55.6 ± 10.2	12.0 ± 3.3	1.6 ± 0.8	0.0032 ± 0.0006
10% EDTA					
Stage D	4	183.8 ± 34.5	28.4 ± 4.5	5.8 ± 1.1	0.0024 ± 0.0001
4% Formaldehyde in 10% EDTA					
Stage D	2	219.9 ± 9.5	42.0 ± 6.9	2.1 ± 0.1	0.0037 ± 0.0010

Postecdysial = Stage A-B cuticle at the leathery phase before becoming brittle with $CaCO_3$.

Stage $C_{3/4}$ = intermolt stage.

Stage D = calcified cuticle covering the pre-ecdysial cuticle.

(F) = 4% formaldehyde only.

Means ± standard deviations.

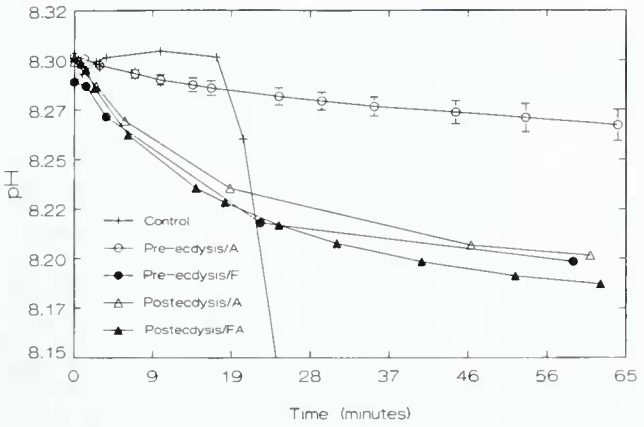

Figure 1. Promotion of $CaCO_3$ crystallization by coupons cut from new cuticle of blue crabs at pre-ecdysis and postecdysis (Stage A–B), following decalcification in either 2% acetic acid (A), 4% formaldehyde (F) to crosslink soluble proteins, or 4% formaldehyde in 2% acetic acid (FA). Crystallization was measured as a downward pH drift, reflecting the removal of CO_3^{2-} from solutions of supersaturated artificial seawater. Promotion of crystallization was indicated by a reduction of the induction period prior to the pH decrease in control solutions. Error bars represent typical standard deviations (n = 3).

lutions with and without formaldehyde. However, there was no significant difference in the ability of coupons to promote crystallization. The ability of coupons to promote crystallization was reduced with prolonged storage (data not shown). Acid-washed pre-ecdysial cuticle did not promote crystallization, presumably because there were few immobilized matrix proteins present. In contrast, crosslinking of soluble protein on pre-ecdysial cuticle promoted crystallization equal to that of formaldehyde-treated or acid-washed postecdysial (stage A–B) cuticle of comparable dry weight (Fig. 1). Coupons of chitin, which should not have protein, did not promote crystallization (Table II), indicating that the protein components may function as nucleating sites.

That the downward pH shifts during the crystallization assays did indeed indicate growth of $CaCO_3$ crystals was verified in three ways: by measurements of calcium on the coupons, by comparison of these measurements to calculations of the amount of carbonate removed from solution, and by the presence of birefringence on the coupons that was acid labile. For example, the coupon that was treated in 4% formaldehyde in 2% acetic acid (Stage D) (Table II) had 20.7 μmole Ca^{2+} deposited on it after crystallization. This compared very well with 20.6 μmole CO_3^{2-} calculated as removed from solution in that same experiment. Finally, birefringence that was present on the coupons after the crystallization experiments was lost following treatment of pieces of coupons with 0.01 *N* HCl, which is consistent with dissolution of $CaCO_3$ crystals.

When a crystal-promoting coupon was suspended in the crystallization solution, the rate of crystal growth was reduced approximately ten fold relative to controls without coupons. The bulk solution was visibly turbid with crystals in controls, but when a coupon was suspended in the solution, crystals were not visible in the bulk solution. SM from crab carapace, when free in solution, inhibited crystallization (Table I); thus, when present in the coupons, some of it may have diffused into the solution, reducing crystal growth rates. In addition, the lowering of the pH of the bulk solution, due to the growth of crystals on the coupons, would itself lead to a lack of precipitation in the bulk medium. The lack of bulk precipitation was not due to formaldehyde diffusing from coupons in that high doses of formaldehyde, when free in solution, did not inhibit crystallization (Table II).

Coupons of chitin or chitosan, which presumably had no protein, did not suppress crystallization in the bulk solution (Tables II, III, respectively). However, Gunthorpe (1989) demonstrated that small coupons of acid decalcified carapace and the membranous layer that underlies the cuticle inhibited crystallization. But when these coupons were extensively washed in distilled water, some

TABLE III

Effect of coupons (5.1 cm^2) of chitosan, glutaraldehyde-treated chitosan, and various peptide-chitosan complexes on $CaCO_3$ crystallization

Complex	n	μmole protein on coupon	Wet weight (mg)	Dry weight (mg)	Induction period (min)	Maximum growth rate (pH/min)
Control	25	—	—	—	15.4 ± 2.5	0.0300 ± 0.0040
Chitosan	5	0	230.4 ± 67.6	71.8 ± 16.6	18.8 ± 4.7	0.0230 ± 0.0060
PolyAsp (MW 11,500)	3	0.040 ± 0.011	182.9 ± 45.4	65.4 ± 10.5	2.9 ± 1.8	0.0036 ± 0.0002
Asp_{20}	4	0.373 ± 0.033	237.4 ± 56.7	64.3 ± 16.7	1.8 ± 0.9	0.0056 ± 0.0009
$Asp_{20}Ala_8$	3	0.320 ± 0.080	189.0 ± 35.9	63.4 ± 14.6	3.1 ± 0.3	0.0042 ± 0.0008
Asp_{40}	4	0.152 ± 0.040	172.0 ± 25.9	57.4 ± 8.2	5.7 ± 1.0	0.0026 ± 0.0001
$Asp_{40}Ala_8$	3	0.126 ± 0.012	180.8 ± 22.0	61.5 ± 2.0	6.2 ± 0.9	0.0022 ± 0.0008

Values are given as mean ± standard deviation for n coupons.

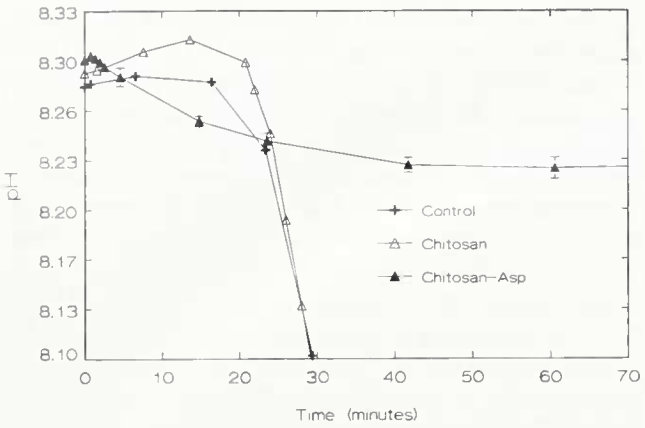

Figure 2. The effect of coupons (5.1 cm^2) of chitosan (without crosslinked peptide or protein) and a peptide-chitosan complex (polyaspartate, MW 11,500, immobilized on chitosan by glutaraldehyde) on $CaCO_3$ crystallization. Error bars represent typical standard deviations (n = 3).

promotion of crystal growth was observed with the washed decalcified carapace present compared to the nearly control levels of crystal growth observed with the washed membranous layer present (Gunthorpe, 1989). Thus, loosely associated protein in the crystal-promoting coupons may have diffused into the solution and inhibited crystal growth. Deposition of crystals was localized on insolubilized protein complexes, giving rise to a slow rate of crystallization that began significantly sooner than observed in controls.

Suspensions of chitosan, a partially cationic surface at pH 8.3, did not promote $CaCO_3$ crystallization (Table III). Various immobilized peptide-chitosan complexes promoted crystallization (Table III; Fig. 2). The presence of an Ala_8 tail on Asp_{20} showed suppression of promotion ($P < 0.01$; balanced, incomplete block design), but this tail did not affect promotion when attached to Asp_{40} (Fig. 3).

The quantities of the various peptides crosslinked to chitosan were statistically the same. Control solutions containing peptides but not chitosan coupons indicated a 4.2 ± 1.6% decrease in the amount of peptides (n = 3) due to interactions with glutaraldehyde after the crosslinking procedure. When the coupon was present, there was a 28.2 to 65.1% decrease in the amount of peptide left in the solution after 1 h. The larger peptides were crosslinked to chitosan to a lesser extent than were the smaller peptides.

As also seen in the studies using the crab carapace system (Table II and Fig. 1), the rate of crystal growth was reduced when coupons with immobilized peptides were suspended in the solution. Crystallization did not occur in the bulk solution in these experiments, rather crystal formation as determined by pH change occurred only on the coupons. This was confirmed by the absence of a pellet of crystals produced by centrifugation of a sample of the bulk solution at 735 × *g*. An equivalent analysis of the bulk solution in control treatments without coupons produced a white pellet of $CaCO_3$ crystals.

Discussion

Inhibition by soluble matrix and by synthetic analogs

Soluble matrix isolated from dorsal crab carapaces, and synthetic peptide analogs of matrix, inhibited $CaCO_3$ crystallization when free in solution. Organic matrices isolated from other organisms are also inhibitors of $CaCO_3$ crystallization when free in solution (Termine *et al.*, 1980; Borman *et al.*, 1982; Doi *et al.*, 1984; Sikes and Wheeler, 1983, 1986; Swift *et al.*, 1986; Wheeler *et al.*, 1988a, b; Wheeler and Sikes, 1984, 1989). Inhibition has been correlated with the affinity of matrix molecules for crystal surfaces and adequate coverage of growth sites on crystal surfaces (Aoba *et al.*, 1984; Wheeler and Sikes, 1989).

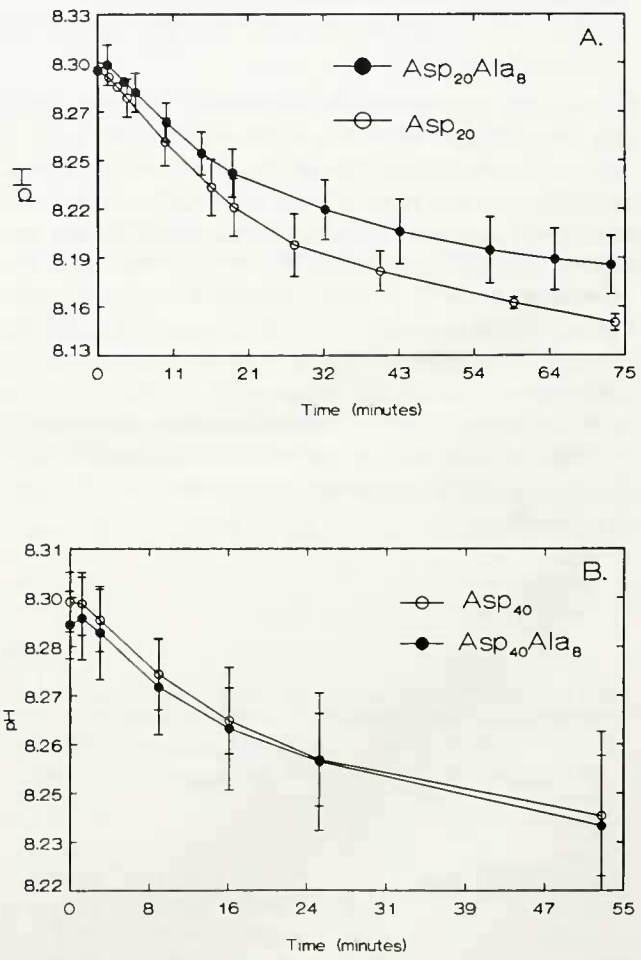

Figure 3. The effect of addition of a polyalanine tail at the C-terminus on polyaspartates of different sizes (A, Asp_{20}; B, Asp_{40}) on promotion of $CaCO_3$ crystallization by immobilized peptide-chitosan complexes.

Immobilized matrix complexes

Decalcified dorsal carapaces of the blue crab (immobilized matrix complexes) and immobilized peptide-chitosan complexes shortened induction periods for crystallization *in vitro*. Shortened induction periods were also observed in collagen systems (Endo and Glimcher, 1988), and similarly, apatite crystals were formed within a shorter period in the presence of proteolipids (Boskey *et al.*, 1988). Shortened induction periods are often considered indicative of an increased rate of nucleation, which can result from a reduction of the energy that is required for nuclei formation (Garside, 1982). A likely cause of such a decrease in nucleation free energy would be a decrease in surficial energy of the nuclei (Wheeler and Sikes, 1989) by the immobilized matrix complex. Crystal nuclei binding by immobilized matrix complexes have been demonstrated (Termine *et al.*, 1981a, b; Addadi and Weiner, 1985, 1986).

The immobilized matrix complex is thought to be formed by an association of soluble matrix with insoluble matrix. The IM framework of crab carapaces is chitin, which, when suspended in a solution supersaturated with respect to calcium and carbonate ions, did not promote crystallization. Further, a derivative of chitin (chitosan) did not promote crystallization when suspended in the solution. With decalcification by acetic acid or EDTA, protein remains associated with the chitin framework (Hunt, 1970; Welinder, 1974; Muzzarelli, 1977; Brine and Austin, 1981). As demonstrated, these latter complexes promoted crystallization. An immobilized matrix complex (whole matrix) from *Nautilus* induced mineral deposition, whereas a preparation of the IM framework alone did not (Greenfield, 1987). To further clarify that SM is the functional molecule, SM was crosslinked to various solid supports. Mineral induction was demonstrated by phosphoproteins crosslinked to collagen (Boskey *et al.*, 1988; Endo and Glimcher, 1988; Linde and Lussi, 1988) or AH-sepharose beads (Lussi *et al.*, 1988; Linde *et al.*, 1989). Soluble matrix from *Mytilus californianus* immobilized on polystyrene films also induced mineral on the film (Addadi *et al.*, 1987). Mineral induction was demonstrated by IM frameworks of other organisms, but the presence of SM was not clarified (Bernhardt *et al.*, 1985; Watabe *et al.*, 1986).

The relative proportions of matrix that was free in solution and that immobilized on the IM framework may correlate with the amount of crystal deposition observed in these studies. Lussi *et al.* (1988) demonstrated that induction by immobilized rat dentin phosphoproteins on AH-sepharose beads can be fully inhibited at high concentrations (>160 μg/ml) of the phosphoprotein when added before the experiment began. A similar phenomenon may, in part, explain the temporal control of calcification in the new cuticle of blue crabs during the molt cycle. The pre-ecdysial layers are not calcified, and only the epicuticle layer is tanned (Roer and Dillaman, 1984; Freeman and Perry, 1985). It may be that the pre-ecdysial cuticle contains more unbound protein that inhibits crystallization, preventing calcification. In contrast, crosslinking of protein onto chitin fibers after ecdysis may promote calcification of the postecdysial cuticle.

Accordingly, pre-ecdysial cuticle that had been acid washed thereby removing SM, did not promote crystallization. With immobilization of pre-ecdysial SM proteins by formaldehyde, crystallization was enhanced. In contrast, postecdysial cuticle promoted crystallization significantly, regardless of the treatment. Roer *et al.* (1988) demonstrated that formaldehyde-treated pre-ecdysial cuticle of fiddler crabs (*Uca pugilator*) induced crystallization, but not to the extent of formaldehyde-treated postecdysial cuticle. Control of calcification was hypothesized to occur by an alteration of the organic matrix resulting in the removal of blocked nucleating sites at ecdysis (Roer *et al.*, 1988).

The immobilized matrix complex can be further characterized by studying specific, synthetic analogs immobilized on a natural IM framework such as chitosan. In so doing, functional groups can be identified. Cationic groups, such as the free amine groups of chitosan, did not promote crystallization. Anionic groups, such as carboxyl groups of aspartate residues, promoted crystallization when immobilized on chitosan supports. In contrast, Addadi *et al.* (1987) demonstrated that polyaspartate (M_r = 6000) adsorbed on polystyrene films did not nucleate $CaCO_3$ crystals on the films, perhaps due to differences in the amounts of polyaspartate immobilized and spatial relationships between the polyaspartate and the different solid supports used in the different studies. However, Addadi *et al.* (1987) also showed that blocking carboxyl groups of protein assemblages from mollusc shells reduced the amount of crystals induced on polystyrene films, and presented other evidence for cooperative influences of SO_4^{2-} and carboxyl groups in promoting crystallization. The importance of anionic groups such as sulfate and phosphate have also been discussed in other studies (Greenfield *et al.*, 1984; Endo and Glimcher, 1988).

In addition to the polyanionic regions, matrix proteins frequently contain substantial hydrophobic domains (Schlesinger and Hay, 1977; Hay *et al.*, 1979; Butler *et al.*, 1983; Schlesinger *et al.*, 1986; Gorski and Shimizu, 1988). The hydrophobic domains are required for complete activity of the proteins as inhibitors of crystallization (Hay *et al.*, 1979, Aoba *et al.*, 1984; Aoba and Moreno, 1990). Therefore, Sikes and Wheeler (1988b) evaluated the effect of attaching a polyalanine domain onto polyaspartate molecules of 15 residues. An enhancement of inhibition of $CaCO_3$ crystal nucleation by polyaspartate-

polyalanine molecules in solution was observed. Perhaps this enhanced inhibition is due to the disruption of the diffusion of lattice ions to the crystal nuclei by the presence of a hydrophobic layer provided by the polyalanine tails of the molecules.

The hydrophobic regions of matrix molecules could also affect their behavior as promoters of crystallization. In this study, therefore, a polyalanine domain of 8 residues was attached to polyaspartate molecules of 20 or 40 residues. These polyanionic-hydrophobic peptides were then crosslinked to the insoluble chitosan coupons so that the effects on $CaCO_3$ crystallization could be evaluated. Attaching the hydrophobic tail to polyaspartate of 20 residues significantly suppressed promotion of crystallization by the immobilized peptide. However, the polyalanine domain attached to polyaspartate of 40 residues had no measurable influence on the promotion of crystallization by the molecules. This is consistent with the observation of Sikes *et al.* (1990), who reported that polyaspartate molecules of about 15 to 20 residues had the optimal size for interaction with $CaCO_3$ crystal nuclei. Although portions of the molecules were occupied by adsorption to crystal nuclei, polyaspartate molecules of 40 residues seemed sufficiently large so as to occupy the zone of diffusion around the crystal surfaces, with or without the additional polyalanine tail.

In the context of the present studies, the results suggest that the negatively charged residues of the $aspartate_{20}alanine_8$ molecules may have been occupied principally with the surface of the crystal nuclei, and the hydrophobic region may have been extruding from the surface, impeding access to the nucleation template. On the other hand, the $aspartate_{40}alanine_8$ molecules may have presented a significant number of negatively charged residues that were not associated with the surface of the crystal nuclei, and were thus available as possible sites for interaction with crystal nuclei, regardless of the presence of the polyalanine domain. In any event, the relative sizes of polyanionic and hydrophobic regions can clearly influence the tendency for a peptide or protein to function as a promoter of crystallization, and one possible function of the hydrophobic zone of matrix molecules is to regulate the promotion of crystallization. Other possible effects of the hydrophobic region, such as changes in secondary structure that might favor peptide interactions with crystals, as described by Addadi and coworkers (1985, 1986, 1987, 1990), also need to be evaluated.

Although the immobilized matrix complexes in this study promoted crystallization, crystal growth rates were suppressed relative to control experiments. That is, crystal growth did not occur in the bulk solution when immobilized matrix complexes were suspended in the solution. However, crystal growth (white, cloudy precipitate) did occur in the bulk solutions of controls and experiments with chitosan coupons without crosslinked peptide or protein. Similarly, Bernhardt *et al.* (1985) observed that mineral was induced on the IM framework, and that no crystals were visible in the solution. Typically, mineral induction has been demonstrated by the presence of crystals on immobilized matrix complexes (Greenfield *et al.*, 1984; Bernhardt *et al.*, 1985; Lussi *et al.*, 1988; Roer *et al.*, 1988) rather than in the solutions. Roer *et al.* (1988) demonstrated, by polarized light microscopy, the presence of crystals grown on decalcified pre- and post-ecdysial cuticle after *in vitro* mineralization. In the present study, crystal deposition on the immobilized matrix complex was observed by acid-labile birefringence of a sample after suspension in the bulk solution. Calcium measurements by atomic absorption spectrophotometry, before and after the experiment, also indicated crystal deposition on the coupon in amounts that were consistent with the downward pH shifts that accompanied the removal of carbonate from solution during crystallization. Deposition of crystal on the immobilized matrix complex and inhibition by diffusible inhibitors such as SM may account for the lack of crystal growth in bulk solutions.

Various mechanisms have been proposed to explain nucleation events. For example, the epitaxial hypothesis requiring spatial matching between immobilized anionic groups and the Ca-Ca distance in the lattice suggests specific amino acid sequences that might function as nucleation sites. Therefore, studies involving formation of peptide-chitosan complexes are underway to test possible mechanisms of matrix-crystal interactions.

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